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A bilayered hybrid microfibrinous PLGA - acellular matrix scaffold for hollow organ tissue engineering

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Key words: bladder tissue engineering; hybrid scaffold; electrospun microfibers; biomaterials, smooth muscle cells; bladder acellular matrix.

Abstract

Various synthetic and natural biomaterials have been used for regeneration of tissues and hollow organs. However, clinical outcome of reconstructive procedures remained challenging due to the lack of appropriate scaffold materials, supporting the needs of various cell types and providing a barrier function required in hollow-organs. To address these problems, we have developed a bilayered hybrid scaffold comprising unique traits of polymeric microfibers and naturally derived acellular matrices and tested its potential for hollow-organ regeneration in a rat bladder model. Hybrid scaffolds were fabricated by electrospinning of PLGA microfibers directly onto the abluminal surface of a bladder acellular matrix. Stability of this bilayered construct was established using modified spinning technique. The resulting 3-dimensional framework provided good support for growth, attachment and proliferation of primary bladder smooth muscle cells. Histological analysis *in vivo* at 4 and 8 weeks post implantation, revealed regeneration of bladder tissue structures consisting of urothelium, smooth muscle and collagen rich layers infiltrated with host cells and micro vessels. Furthermore, hybrid scaffolds maintained normal bladder capacity, whereas BAM-recipients showed a significant distension of the bladder. These results demonstrate that this adaptable hybrid scaffold supports bladder regeneration and holds potential for engineering of bladder and other hollow-organs.

1. Introduction

Hollow organs such as urinary bladder, urethra, ureter, esophagus, intestine, uterus, vagina and blood vessels, generally share a similar functional anatomy consisting of luminal endothelium surrounded by smooth muscle cells [1]. Pathological changes in their functional anatomy caused by trauma, spinal cord injury, malignancy, infection, inflammation and congenital abnormalities may result in dysfunction and profoundly affect the patients' quality of life [2-4]. Many patients, including children, suffer from urinary bladder pathologies requiring reconstructive procedures [5, 6]. Gastrointestinal segments are the gold standard for bladder reconstruction, however this procedure is associated with significant long-term complications including urinary tract infection, metabolic abnormalities, stone formation and malignancies [7, 8]. Therefore, there is a strong clinical need for alternative biomaterials for these reconstructive procedures [9].

Tissue engineering (TE) approaches provide potential strategies to develop biological substitutes which can restore and maintain normal functional anatomy [10]. In TE, biomaterials play a central role by creating a pre-configured three-dimensional microenvironment that supports attachment, proliferation, migration and differentiation of transplanted or regenerating resident cells and promotes cell-cell interactions, extracellular matrix deposition and new tissue development [11, 12]. Moreover, biomaterials should address the needs of various cell types involved in the regeneration process. The ideal biomaterial should possess desired properties concerning biodegradation and display a reliable biological performance without inducing a host immune response. Thus, the selection and design of ideal biomaterial mimicking the native architecture of the target tissue remains critical to generate functionally equivalent substitutes.

Two main classes of materials, i.e., acellular matrices [13, 14] and biopolymers [15, 16], have been used for the regeneration of hollow organs. Acellular matrices derived from donor tissue (e.g., bladder submucosa) offer excellent trophic support as they are naturally enriched with a wide range of growth factors, thereby stimulating new tissue growth and providing attachment sequences that

facilitate cell-material interactions and the maintenance of the functional phenotype [17, 18]. In addition, they also prevent permeation of luminal contents into the abdominal cavity [19]. These matrices undergo biodegradation upon implantation and are easily remodelled by biological activity of transplanted or ingrowing cells [19]. However, these dense materials cannot harbour a high density of smooth muscle cells (SMCs) [10]. In contrast, synthetic polymer, i.e., polyglycolic acid (PGA), polylactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA) based fibrous scaffolds can be fabricated reproducibly with the desired and controllable mechanical and degradation properties, porosity as well as topographies providing guidance structures [20, 21]. However, the biological competence of synthetic polymer based fibrous scaffolds is limited given their lack of both trophic support and natural barrier function of luminal endothelium.

Both of the above mentioned materials have desirable traits exclusive of each other and none of the scaffolds alone can mediate the functional regeneration of the defective hollow organ.

A functionally equivalent substitute for hollow organ tissue engineering should possess both tropic and topographical functions, while mediating barrier function. The development of such a pre-configured composite biomaterial construct providing the native architecture of the target organ might enhance the clinical outcome of tissue engineered reconstructive procedures. In this line of study, we have previously reported on a composite biomaterial fabricated by suturing a collagen matrix to PGA polymers which we tested in an *ex-situ* mice model. A major drawback of this scaffold is the potential leakage of urine through the holes created by threaded collagen stitches (1). The development of a stable composite material without the use of any external bonding materials may offer several advantages such as easy surgical handling and watertightness.

Therefore, we have developed a bilayered hybrid scaffold by electrospinning of polymer microfibers directly on a bladder acellular matrix and evaluated its biological performance *in vitro* and *in vivo* for the engineering of hollow organs using a rat bladder model. The stability of the

bilayered scaffold was established by modifying the spinning technique and tested in aqueous buffer. Morphological features of the hybrid scaffold were characterized by scanning electron microscopy (SEM). SMCs, which are representing the main cell types of hollow organs, were seeded into microfibrinous layers of hybrid scaffold and analysed *in vitro* for cell attachment and proliferation. Furthermore, the hybrid scaffolds were implanted into a rat bladder and the biological performance was evaluated at 4 and 8 weeks post-operatively by analysing anatomical and functional outcomes.

2. Materials and Methods

2.1. Preparation of bladder acellular matrix

Urinary bladders from adult pigs were subjected to a multi-step detergent washing procedure based on an established protocol [1]. Briefly, the muscle layer from porcine bladder tissue was micro-dissected and removed leaving the lamina propria, also referred to as the bladder submucosa. All subsequent washes were performed on an elliptical shaker at 4°C. The bladder submucosa was treated with distilled water for 2 days, changing the water twice a day to lyse the cells in the tissue. Subsequently, the tissue was treated with Trypsin 0.05% for 1 hour. After blocking with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) containing 10 % fetal bovine serum (FBS) over night the tissues were treated with 1% Triton X100 and 0.1% ammonium hydroxide for 7 days, changing the detergent daily. Thereafter, the collagen matrix was rinsed with phosphate buffered saline (PBS) for two days. Representative samples of freshly processed matrices were analyzed by hematoxylin eosin staining and DNA quantification by spectrophotometry to confirm the removal of all cellular constituents. The detergent wash was repeated if necessary to ensure complete extraction. The bladder acellular matrix (BAM) was stored in hydrated state at 4°C.

2.2. PLGA-BAM composite scaffold production

The microfibrinous scaffold was fabricated on the abluminal side of the BAM by electrospinning. An 8% PLGA (RG 85:15, Boehringer Ingelheim, Germany) polymer solution (wt/wt) in chloroform

(Sigma Aldrich, Switzerland) was loaded into 2 ml syringe (B. Braun Melsungen AG, Germany) fitted with a spinning head consisting of a blunt end stainless needle (1 mm inner diameter and 0.3 mm wall thickness, Angst & Pfister AG, Zurich, Switzerland). The polymer solution supply was adjusted to a flow rate of 1.6 ml/h using a programmable syringe pump (AL1000 Aladdin, World Precision Instruments, Germany) and horizontal spinning was performed with an accelerating voltage of 12 kV supplied by a high voltage power supply (Glassman High Voltage Inc., High Bridge, NJ, USA). The BAM was pre-fixed on a cylindrical collector (length: 100 mm, diameter: 80 mm, wall thickness: 5 mm) by placing the luminal side of the BAM onto an aluminum foil-covered mandrel. The collector speed was set to 50 rpm and positioned at a distance of 20 cm from the tip of the needle. Fibers were electrospun directly onto the abluminal side of the BAM at room temperature (RT) and pressure. Three different spinning procedures were employed in order to define the optimal method to produce a stable hybrid scaffold. Those include continuous spinning of PLGA microfibers on dry BAM, continuous spinning of PLGA microfibers on wet BAM and layer-by-layer spinning of PLGA fibers on continuously rehydrated BAM. In contrast to the spinning on wet BAM, the rehydrated BAM was maintained hydrated throughout the spinning process by 20 min re-hydration with 100 mM PBS every hour during the layer by layer spinning procedure.

2.3. Stability of composite scaffold in aqueous buffer

The stable attachment of electrospun microfibers to the abluminal surface of the acellular matrix under aqueous conditions is an important feature of our hybrid scaffold allowing pre-implantation cell seeding and easy surgical handling. To test the attachment of electrospun fibers on the surface of acellular matrix and its stability, BAM-PLGA composite scaffolds were fabricated using a 5-(aminoacetamido) fluorescein (Invitrogen, Switzerland) (1mg/ml) blended PLGA polymer solution. The 3 different techniques mentioned above were used for the production of electrospun scaffolds and full thickness scaffolds were resized into squares of 1 cm². Hybrid scaffold samples were incubated in PBS buffer at RT under mild shaking (50 rpm) and observed every 24 hrs over 7 days

using an epifluorescence microscope (Zeiss Axiovert 200M, Germany) to verify whether the fluorescent fibrous PLGA layer was attached to the BAM, fully detached or if individual fibers remained attached on the BAM surface. Three parallel experiments were performed and 10 samples were taken for each condition in all the experiment. Hybrid scaffolds with partly or fully detached microfibrinous layer were considered unstable. Scaffold stability was measured by using following method. Percent stability = Number of intact scaffolds x 100/Total number of scaffolds.

2.4. Scanning electron microscopy

Scaffold morphology, fiber diameter, interfiber space and scaffold thickness were analyzed by SEM. To this effect, scaffolds were dried overnight in a vacuum chamber and sputter coated with gold using a Hummer V sputtering system (Technics Inc., Baltimore, MD) at 50 mA to obtain a 10 nm coating. Samples were analysed using a Zeiss SUPRA 50 VP (Zeiss, Cambridge, UK) SEM operated at an accelerating voltage of 6 kV at various magnifications between 100 x and 20k x. ImageJ 1.31v software (National Institutes of Health, Bethesda, USA) was used for the quantitative analysis of the fibrous scaffolds and randomly selected fibers (n=20) from different locations were chosen for size measurement.

2.5. Porosity measurement

Electrospun PLGA microfibrinous scaffolds were dried in a vacuum oven and resized into squares of 1 cm². The apparent density of electrospun PLGA polymer scaffolds were determined gravimetrically using the weight measurements of the precisely cut scaffolds of defined area and thickness. The scaffold dimensions were measured from SEM micrographs of the electrospun PLGA scaffolds. Apparent density and porosity were calculated using the following equations as described previously[22, 23].

$$PLGA_{\text{apparent density}}[g \cdot cm^{-2}] = \frac{10 \cdot PLGA_{\text{mass}}[mg]}{PLGA_{\text{thickness}}[\mu m] \cdot PLGA_{\text{area}}[cm^2]}$$

$$PLGA_{\text{porosity}}[\%] = \frac{PLGA_{\text{bulk density}}[g \cdot cm^{-2}] - PLGA_{\text{apparent density}}[g \cdot cm^{-2}]}{PLGA_{\text{bulk density}}[g \cdot cm^{-2}]} \cdot 100$$

Note: Bulk density of the PLGA is 1.15 g/cm³ [23]

2.6. Smooth muscle cell isolation, proliferation and seeding

Primary SMCs were harvested from rat bladders. After removal of the urothelium by mechanical scraping the muscle layer was cut in small pieces of 1x1 mm and placed into a 10 cm culture dish. After 20 min Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% FBS was carefully added. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Media was replaced every 3-4 days. The cells were expanded and characterized by immunohistochemistry and fluorescence-activated cell sorting (FACS). Prior to seeding, the hybrid scaffolds (10x10 mm) were incubated in DMEM supplemented with 20% FBS, 3% Penicillin-Streptomycin and 3% Fungizone at 4°C overnight. The samples were then rinsed in PBS and placed in a 12 well dish. SMCs at passage 3-4 were trypsinized and counted. For each scaffold 8x10⁵ cells were suspended in 40 µl of culture medium, seeded onto the fiber side of the scaffold and incubated at 37°C for 2 hours. Subsequently 2 ml culture medium was added to each well and the scaffolds returned to the incubator for 8 days with a daily medium change.

2.7. Immunocytochemistry

To confirm the SMC phenotype, primary rat bladder SMCs grown on chamber slides (Nunc., Roskilde, Denmark) were fixed with 4% paraformaldehyde (PF) for 10 min. After rinsing with PBS

the cells were permeabilized with 0.5% Triton X-100 for 7 min and then blocked with 1% protease free BSA for 30 min. Cells were treated with primary antibodies for alpha-actin (1:100, Sigma-Aldrich, St. Louis, MO, USA), smoothelin (1:100, Santa Cruz, Santa Cruz, CA, USA) and calponin (1:100, Sigma-Aldrich, St. Louis, MO, USA), at RT for 1 h. Subsequently cells were incubated with the corresponding secondary anti-mouse antibody (Anti-Rabbit IgG Cy3, 1:100, Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Nuclei were stained with DAPI (1:500, Sigma-Aldrich, St. Louis, MO, USA) actin filaments with Phalloidin (1:100, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h. Cell samples without addition of primary antibody served as negative controls.

2.8. Fluorescence-activated cell sorting

The SMC phenotype and homogeneity of the culture were investigated by FACS. Cell suspensions were incubated with primary antibodies for alpha-actin (mouse monoclonal, 1:100, Sigma-Aldrich, St. Louis, MO, USA), smoothelin (rabbit polyclonal, 1:100, Santa Cruz, Santa Cruz, CA, USA) and calponin (mouse-polyclonal, 1:100, Sigma-Aldrich, St. Louis, MO, USA). Cells were then incubated with the corresponding FITC labelled polyclonal secondary antibody and subsequently analysed by flow cytometry (BD FACSCanto™ Flow Cytometer, BD Bioscience, Belgium). Specificity of labeling was demonstrated with the corresponding isotype control for mouse IgG1 (1:100, Santa Cruz, Santa Cruz, CA, USA) and Rabbit IgG1 (1:100, Invitrogen, Basel, Switzerland).

2.9. Smooth muscle cells growth and proliferation on hybrid scaffold

Three-dimensional cell growth on the biomaterial was quantified using a colorimetric assay with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt reagent (WST-1, Roche, Mannheim, Germany). SMCs were seeded at a density of 8×10^5 cells/cm² onto the hybrid scaffolds in 12-well plates. At three different time points (2, 4 and 8 days) 2 ml culture medium plus 200 µl WST-1 was added to each scaffold and incubated at 37°C for 4 h. Cell viability was measured in the supernatant at 450 nm in a scanning multiwell spectrometer (KC4™ Software

BioTek Instruments, Inc. Luzern, Switzerland). BAM alone served as a control. For direct visualisation of the growing SMCs, seeded scaffolds were fixed with 4% PF for 10 minutes, permeabilized with 0.5% Triton X-100 for 7 min and blocked with 1% protease free BSA for 30 min. Cells were then incubated with DAPI (1:500, Sigma-Aldrich, St. Louis, MO, USA) and Phalloidin (1:100, Sigma-Aldrich, St. Louis, MO, USA) for 2 h. After rinsing with PBS, the samples were mounted on a coverslip in DABCO-mounting medium (Sigma-Aldrich, St. Louis, MO, USA) and imaged by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

2.10. Surgical implantation of hybrid scaffolds

All procedures used in this study followed the Care and Use of Laboratory Animals and were approved by the Veterinary Office of the Canton of Zurich, Switzerland. A total of 18 adult female Lewis rats (~200 g) underwent partial cystectomy and subsequent bladder reconstruction. The rats were randomized into two groups. Group 1 received the hybrid scaffold (n=8), Group 2 served as a control, receiving BAM alone (n=8). 4 healthy rats served as controls for morphological and histological investigations.

All surgeries were performed under general anaesthesia, using 2% isoflurane. The lower abdomen was shaved and disinfected. The urinary bladder was exposed through a lower abdominal incision and partial cystectomy (50%) was performed. The bladder was reconstructed using the scaffold anastomosed to the remaining host bladder with running 7-0 absorbable vicryl sutures. To identify the junction between native tissue and graft, the margins were marked using 7-0 non-absorbable polypropylene sutures. The bladders were then filled with normal saline to prove water tightness. The omentum was intraoperatively placed on top of the patch and the abdomen was closed in layers using absorbable running sutures.

After cystometric bladder capacity measurement (see paragraph 2.13) at 4 and 8 weeks post-operatively, the engineered bladder tissues (n=4) were retrieved from each group and inspected

macroscopically. In order to assess graft shrinkage, the non-absorbable sutures were identified and the graft area was measured.

2.11. Histological analysis

For histology, engineered bladder tissue was fixed with 4% formaldehyde and paraffin-embedded. Bladder sections (5 μ m) were stained by hematoxylin-eosin and Masson's trichrome staining for histomorphological assessment. Histological images were captured (Leica Microsystems, Wetzlar, Germany) at various magnifications. Bladder sections were deparaffinised and processed for specific markers of urothelium and smooth muscle. Non-specific bindings were blocked using 5% BSA, 0.3% Triton X100 in PBS for 1h. Sections were incubated overnight with polyclonal anti-pancytokeratin AE1/AE3 antibody (1:50, Abcam Inc., USA) and anti-smoothelin antibody (1:100, Santa Cruz, Santa Cruz, CA, USA). Following washing with PBS buffer, the secondary antibody, Fluorescein anti-rabbit IgG (1:1000, Vector Laboratories, Burlingame, CA, USA), was applied at RT for 1 h. After rinsing with PBS, the slides were mounted with DABCO-mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Analysis was carried out using a Leica DM6000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.12. Bladder capacity measurement

The measurements were performed at the time of cystoplasty and 4 or 8 weeks after implantation of the hybrid scaffold. Under isoflurane anaesthesia a 26 GA catheter (BD Insite, Becton Dickinson SA, Madrid, Spain) was inserted into the bladder. The bladder was emptied and the tubing connected to a physiological pressure transducer (AD Instruments, Germany). Prior to recording, the isoflurane level was reduced to allow for standardisation of the anaesthetic depth. Saline at RT was infused at 10 ml/h via an infusion pump and intravesical pressure was recorded. Maximal bladder capacity was determined as the volume at which leakage occurred or at which a pressure of 40 cmH₂O was reached.

2.13. Statistical analysis

SPSS 18.0 (SPSS Inc., Chicago, IL) was used for data analysis. In this report all data were expressed as mean and standard error of the mean. To assess the difference between the groups one-way analysis of variance (ANOVA) with a Bonferroni *post-hoc* test or an independent student-t-test was performed for the numeric data. A p-value of < 0.05 was considered significant.

3. Results

3.1. BAM characterization

Prior to cell seeding, the acellularity of the freshly processed BAM was confirmed histologically and by DNA extraction (Fig. 1). Light microscopy showed an intact fibre framework with no evidence of remaining nuclei and DNA extraction verified the acellularity of the BAM with remaining DNA concentrations below 30 ng/mg.

3.2. PLGA-BAM hybrid scaffold production and characterization

Full thickness bilayered hybrid scaffold consisting of PLGA microfibrous scaffold and BAM were fabricated successfully using electrospinning technology. Electrospinning of 6 ml of PLGA (8%) polymer solution on dry BAM, wet BAM without rehydration during spinning process and continuously rehydrated BAMs resulted in production of 3 different hybrid scaffolds.

The analysis by SEM revealed no visible differences among the 3 different scaffolds in terms of morphological features (Fig. 2). SEM Images revealed bead less, porous structures of the electrospun full thickness scaffold ($180\ \mu\text{m} \pm 20\ \mu\text{m}$) and homogenous deposition of randomly oriented PLGA microfibers with a mean diameter of $4.5\ \mu\text{m} \pm 0.25\ \mu\text{m}$. This indicates that conditions of the BAM have no influence on the morphological features of the electrospun PLGA microfibers. In contrast, the stability of the hybrid scaffolds was significantly affected by the

spinning procedure. Cross sectional analysis revealed stable attachment of different layers of hybrid scaffold produced by layer-by-layer spinning on rehydrated BAM. In contrast, microfibrillar layer detached completely from acellular matrices in the scaffolds fabricated by continuous electrospinning on dry or wet BAM (Fig. 2) indicating the importance of maintaining BAM hydration on the integrity of hybrid scaffold. Furthermore, images in fig. 3A demonstrate the influence of the spinning process on fiber attachment in 3 different scaffolds at various time points as measured by the signals emitted by fluorescein labelled PLGA microfibers. In case of continuous spinning on dry BAM, fibers were poorly attached at day1 (10%) and completely detached by day 2 (Fig. 3A and 3B). Hybrid scaffolds with continuous spinning on wet BAM showed moderate stability i.e., 40% at day 1, but significantly declined to 10% by day 7. Microscopic analysis of the acellular matrix of unstable scaffolds revealed no remnant fibers on the surface, indicating that the PLGA-BAM interface is critical for the stability of the hybrid scaffold. Scaffolds that maintained a hydrated state of BAM through continuous rehydration and layer-by-layer spinning showed significantly improved (90%) attachment of electrospun fibers to acellular matrix at all time points when compared to the other two production procedures (Fig. 3A and 3B).

These results indicate that stable attachment of electrospun fibers on the BAM surface requires maintenance of a hydrated state. Therefore, layer-by-layer spinning with continuous rehydration procedure was chosen as the best method for the fabrication of stable hybrid scaffolds for further *in vitro* and *in vivo* evaluation. The porosity of this scaffold was calculated to be $70.8 \pm 4.2 \%$, showing that the scaffolds were highly porous with fine fibres imparting a large surface area and interfiber space was in the range of $12.25 \pm 4.75 \mu\text{m}$ creating large pore size.

3.3. In vitro evaluation of hybrid scaffold

Immunostaining and FACS analysis of primary bladder SMCs used for seeding confirmed their cell type and homogeneity of the cultures (Fig. 1C-E). Flow cytometry revealed that $92 \pm 3\%$ of the cells were positive for alpha-actin, $96 \pm 1\%$ for smoothelin and $78 \pm 7\%$ for calponin. The results of cell

growth and proliferation on the scaffolds are shown in Fig. 3. The WST-1 assay 2, 4 and 8 days after seeding revealed significantly enhanced cell growth on hybrid scaffolds (0.96 ± 0.14 , 1.23 ± 0.11 and 1.93 ± 0.34 , respectively) compared to BAM alone (0.29 ± 0.04 , 0.29 ± 0.03 and 0.59 ± 0.15 , respectively) (Fig. 1F). Immunostaining of seeded SMCs showed an increasing number of cells on the PLGA scaffold and infiltration into the fibrous scaffold was observed over time. SMCs were well attached to the fibers, maintaining their characteristic spindle-phenotype shape.

3.4. In vivo evaluation of hybrid scaffold

Due to postoperative complications two animals had to be replaced in this study. One rat developed a lethal urosepsis and one died as a consequence of leakage at the site of anastomosis due to technical error. All other rats recovered well from the procedure.

At the time of sacrifice, all scaffolds were integrated into the native bladder and there were no signs of inflammation, necrosis or soft tissue calcification. Some adhesions were noted between the hybrid biomaterial and the omentum, which was intraoperatively placed on top of the patch. At 4 weeks the average patch size was $65.1 \pm 2.7 \text{ mm}^2$ in the hybrid scaffold group and $73.7 \pm 6.5 \text{ mm}^2$ in the BAM group. At 8 weeks the average patch size was $56.2 \pm 4.5 \text{ mm}^2$ and $53.75 \pm 1.6 \text{ mm}^2$, respectively. In the BAM group the shrinkage was statistically significant ($p=0.006$) but not in the hybrid scaffold group ($p=0.195$) (Fig. 4).

Histological examination of the engineered bladder wall in all groups revealed a distinct multilayered architecture containing all bladder wall components (Fig. 5A). The whole inner surface of the graft in both groups was covered by a uniform anti-pancytokeratin AE1/AE3 positive urothelial lining. SMCs were present in all scaffold sections, typically aggregated into a suburothelial layer consisting of small irregular muscle bundles confirmed by smoothelin positive staining. Submucosal collagenous tissue with mixed cellular infiltration and numerous micro vessels was detected in both groups. Polynucleous cellular infiltration (giant cells) was found in the

fibrous PLGA layer at both time points. The thickness of the engineered bladder wall was measured in tissue sections derived from scaffold neo-tissues retrieved 4 and 8 weeks after implantation (Fig. 5B). While native bladder wall had a thickness of $1040 \pm 18 \mu\text{m}$, the retrieved samples at 4 weeks had a thickness of $1245 \pm 117 \mu\text{m}$ in the hybrid scaffold group and $1115 \pm 84 \mu\text{m}$ in the BAM group. After 8 weeks the thickness was $1375 \pm 348 \mu\text{m}$ and $904 \pm 139 \mu\text{m}$, respectively, showing an increased regeneration in the groups reconstructed with hybrid scaffold, although differences appeared not to be significant.

The preoperative bladder capacity was $0.55 \pm 0.05 \text{ ml}$, here referred to as normal control. After 4 weeks, the capacity measured $0.77 \pm 0.06 \text{ ml}$ in the hybrid scaffold group and $1.43 \pm 0.39 \text{ ml}$ in the BAM group, after 8 weeks $0.69 \pm 0.14 \text{ ml}$ and $1.46 \pm 0.36 \text{ ml}$, respectively (Fig. 4D). Together these results demonstrate that bladders reconstructed using hybrid scaffolds maintained normal bladder capacity throughout the study period, while bladders reconstructed using BAM showed significant distension and thinning of the bladder wall.

Discussion

The ideal scaffold for the regeneration of a urinary bladder and other hollow organs needs to fulfil specific requirements pertaining to 3-dimensional architecture, micro-environmental needs of the various cell types of the hollow organ wall, diffusion barrier function and structural integrity of the hollow organ. Towards this goal, we have developed a technique to manufacture a hybrid scaffold by direct electrospinning of PLGA microfibers on bladder acellular matrix. We demonstrated that direct spinning of PLGA fibers on BAM leads to a scaffold with bimodal morphological features similar to the configuration of the native bladder wall. Without the use of stabilizing additives and threaded stitches, these hybrid scaffolds allow easy handling and remain appropriate for surgical implantation. This bilayered hybrid scaffold is characterized by well defined and reproducible microstructures of fibrous scaffold and underlying acellular matrix, combining the advantages of the two different materials used. Among all electrospinning procedures applied, only layer by layer spinning on continuously hydrated BAM resulted in a stable hybrid scaffold. This can be explained by the physiological state of the collagen rich BAM, which was maintained by continuous rehydration, thereby possessing more adhesive properties compared to other conditions applied to BAM.

Several studies used electrospun scaffolds consisting of micro/nano scale polymeric fibers mimicking target tissue architecture and analyzed cellular interactions fibrous-microenvironment *in vitro* [4, 24-28]. For urological tissue regeneration, Kundu et al. fabricated synthetic composite biomaterial and tested *in vitro* using bladder tissue cells [29]. All these studies resulted in better understanding of cell-material interactions. However, these composite materials are not ideal for hollow organ tissue engineering, as they don't possess trophic support and natural barrier function required for maintaining luminal contents. Eberli et al. described the manufacturing of a bilayered scaffold by suturing a collagen matrix to polyglycolic acid (PGA) polymer with collagen threaded stitches [1]. Scaffolds were implanted subcutaneously in a mouse model have been shown to be non-cytotoxic and possessed adequate physical and structural characteristics. However, suturing of

polymers may compromise the barrier function which is indispensable for bladder reconstruction and these constructs should be further investigated in an *in situ* cystoplasty model.

To meet the specific microenvironmental needs of the different cell types of the bladder we utilized two well-established materials for manufacturing a bilyered hybrid scaffold. Collagen matrices and PLGA have gained approval by the US Food and Drug Administration (FDA) for a various clinical applications [10, 30]. In addition, both materials have been used as single layers in bladder reconstruction and they have demonstrated excellent support for cell growth both *in vitro* and *in vivo* [13, 15, 16, 31-33]. BAM was chosen to improve stability, to provide barrier function and to promote the attachment of urothelial cells. In order to rule out unwanted side effects of freeze-drying, we have maintained the BAM in a hydrated state at 4°C [34, 35]. The purpose of the PLGA fibrous network is to improve structural support for cellular infiltration. SMCs seeded on PLGA fiber scaffolds showed higher growth and proliferation rate than SMCs seeded on BAM alone. These results suggest that the 3-dimensional fibrous scaffold with interconnected pores and high surface area to volume ratio provides better support for attachment and proliferation of SMCs.

Previous studies demonstrated that the implantation of acellular matrix grafts alone promotes the ingrowth of urothelium, smooth muscle, blood vessels and nerves [16, 32, 36]. Comparably the *in vivo* data of this study demonstrated that the hybrid scaffolds were able to provide a favourable environment for re-growth of UCs and SMCs leading to a multilayered architecture with all bladder wall components present. It has been shown that the formation of a urothelial lining is not only important for the barrier function [37] but also mandatory for structured regeneration of the other bladder wall components [38]. Furthermore, infiltration of SMCs is critical for the successful regeneration of functional tissue after implantation of engineered constructs. The regeneration of a suburothelial SM layer as observed in both groups in our study is consistent with the findings of other researchers, who have noted that newly developed SM appears different from native detrusor in terms of orientation and bundle formation [32, 36, 39]. SM bundle formation was mainly seen

close to the acellular graft indicating an interaction of developing SMC layers and the urothelium. Further, vascularization of the graft is mandatory to ensure the metabolic needs of the developing tissue and its integration within the surrounding native tissue. Vascularization was detectable in this area in all samples indicating the suitability of the hybrid scaffold for neo-tissue development [28]. Taken together, these results indicate that this hybrid scaffold possesses adequate regenerative potential to promote regeneration of bladder wall tissue and provide basis for further improvement of the hybrid scaffold's properties (porosity and smooth muscle cells seeding) towards functional bladder tissue regeneration.

Consistent with previous reports [40], [41] HE staining revealed inflammatory cells in the regenerated bladder. Natural collagen matrices such as Small Intestine Submucosa (SIS) and BAM generate a minimal inflammatory reaction following implantation whereas synthetic biomaterials can trigger a more prominent response due to acid by-products [40-42]. Eight weeks after implantation we encountered shrinkage of the scaffold size of 14% in the hybrid scaffold group and 27% in the BAM group. However, there did not appear to be any resultant bladder dysfunction secondary to the loss of surface area. Bladder capacity measurements demonstrated that the capacity of bladders augmented with hybrid scaffold was statistically comparable to normal bladders whereas the BAM group showed significantly increased bladder capacities. PLGA fibers in the hybrid biomaterial serve as a supporting framework preventing overdistension and diverticula formation, whereas BAM alone seems to slacken, leading to ballooning of the reconstructed area.

Conclusion

The data presented in this study demonstrate the feasibility of developing hybrid scaffold combining unique functions of electrospun microfibrinous network and underlying natural acellular matrix. The hybrid scaffold provided natural barrier function and supported microenvironmental needs of the various cell types of bladder wall as evidenced by urothelial and smooth muscle regeneration. Functional measurements showed sustained normal bladder capacity in hybrid

scaffold recipients, whereas BAM recipients displayed significant bladder distension. Furthermore, this hybrid scaffold could offer an adaptable platform for delivering cells, growth factors and topographical guidance factors, and holds potential for tissue engineering of bladder and other hollow organs. Future studies will focus on evaluation of improved porosity and pre-seeding of hybrid scaffold with SMCs on long-term functional bladder tissue regeneration in animals.

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Figure captions:

Figure 1. BAM production, bladder smooth muscle cell characterization and cellular growth on hybrid scaffold.

Acellularity of bladder acellular matrix was proven by H&E staining (A), showing a dense fibre framework without remaining nuclei or cellular fragments. Confirmation of acellularity of processed BAM by DNA quantification compared to normal bladder tissue (B). Primary rat bladder smooth muscle cells stained with antibodies for alpha actin (C) and smoothelin (D) (red). Cell nuclei were co-stained with DAPI (blue). FACS analysis showing representative expression of alpha actin, smoothelin and calponin evidentiary for differentiated (contractile) state of smooth muscle cells (E). WST-1 assay showing SMCs proliferation on different scaffolds at various time points (F) (* $p < 0.005$).

Figure 2. SEM micrographs of bilayered hybrid scaffold produced by electrospinning of PLGA microfibers directly onto bladder acellular matrix (BAM). 3 different hybrid scaffolds were produced as a result of various conditions applied to BAM during spinning procedure. Surface morphology displayed homogenous deposition of randomly oriented PLGA fibers with fine surface structure and interconnected pores between the fibers in all the scaffolds. Cross sectional images revealed influence of spinning procedure on the integrity of two different layers in composite scaffold. Red colour bar indicates BAM, blue colour bar indicates microfibrous scaffold and white colour arrow indicates gap between these two layers. The two layers of the hybrid scaffold remained well attached when the BAM was continuously rehydrated during the spinning procedure.

Figure 3A. Attachment and stability of the two different layers of hybrid scaffolds in aqueous buffer. Microscopic analysis of influence of spinning procedures on attachment of PLGA microfibers onto surface of acellular matrix. Fibers attachment was confirmed at different time points as an indicative of signals emitted by fluorescence blended PLGA microfibers.

Figure 3B. Quantitative analysis of stability of hybrid scaffolds at different time points (* $p < 0.001$).

Figure 4. Cystoplasty with hybrid scaffold intraoperative view (A) and after 8 weeks *in vivo* (B). Arrow indicating marked border between native bladder and scaffold. Scaffold diameter (C) and bladder capacity after 4 and 8 weeks (D) implantation compared to normal bladder values (* $p < 0.005$).

Figure 5A. Histology of the hybrid scaffold and control scaffold (8 weeks after implantation) compared to normal bladder wall. Hematoxylin and Eosin stain (A) showing a normally structured mature urothelium and giant cell reaction in the PLGA fiber layer. Masson's trichrome stain (B) showed a collagen rich layer as well as a subepithelial muscle layer. (C) Immunohistochemistry confirmed the smooth muscle cell phenotype (anti-

smoothelin) and showed the presence of blood vessels mainly in the hybrid scaffold group. Uroendothelium and smooth muscle layers are indicated by arrow and asterisk respectively. Scalebar 100 μm .

Figure 5B. Bladder wall measurements at 4 and 8 weeks after implantation.